Journal of Medical Virology 52:262–269 (1997)

Evaluation of Multiple Antibodies to Epstein-Barr Virus as Markers for Detecting Patients With Nasopharyngeal Carcinoma

Mei-Ying Liu,[1] Yen-Liang Chang,[2] Jeng Ma,[3] Huey-Lang Yang,[3] Mow-Ming Hsu,[2] Chien-Jen Chen,[4] Jen-Yang Chen,[1]* and Czau-Siung Yang[1]

[1]*Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China*
[2]*Department of Otolaryngology, College of Medicine, National Taiwan University, Taipei, Taiwan, Republic of China*
[3]*Graduate Institute of Epidemiology, College of Public Health, National Taiwan University, Taipei, Taiwan, Republic of China*
[4]*Molecular Biology Division, Development Center for Biotechnology, Taipei, Taiwan, Republic of China*

Five serological tests were assessed for their sensitivity for screening and early detection of nasopharyngeal carcinoma (NPC). The tests included the detection of antibodies to various gene products of EBV: viral capsid antigen (VCA) using an indirect immunofluorescence assay (FA), DNase using an activity neutralisation test (NT), DNase using an enzyme-linked immunosorbent assay (ELISA), DNA polymerase (DP) using NT, and major DNA binding protein (MDBP) by ELISA. Sera from 100 NPC outpatients and 20 NPC patients, who were detected in a prospective study, were examined. The results showed that levels of antibody to DNase detected by ELISA and to DP detected by NT and the positivity rate for VCA by FA increased with NPC stage. More species of EBV antibody became detectable as NPC progressed. The detection of anti-MDBP antibody by ELISA was suitable for screening for NPC. Anti-DP antibody detected by NT was a valuable marker both for early detection and prognosis of NPC. Detection of anti-DNase antibody by ELISA was the most sensitive method for detection of NPC. No single test was sufficient to detect all the NPC patients and a combination of anti-DNase by ELISA with other tests are recommended to identify NPC patients. ***J. Med. Virol. 52:262–269, 1997.***

KEY WORDS: nasopharyngeal carcinoma; screening; early detection; EBV antibodies

INTRODUCTION

The highest incidence of nasopharyngeal carcinoma (NPC), which is a rare malignant neoplasm in most countries, is observed in the Chinese population, especially in southern Chinese males [Shanmugaratnam, 1982]. In Taiwan, NPC is one of the most common cancers [Yeh, 1985] and has a significant socio-economic impact due to the relatively early age of onset. Delayed diagnosis usually results in a low survival rate. However, 10-year survival rates for NPC patients may be as high as 77% if treatment is carried out promptly at stage I [Hsu et al., 1982]. In contrast, patients receiving treatment at stage V never live longer than 2 years. The development and evaluation of assays for the early detection of NPC are essential if overall survival rates are to be improved.

Extensive evidence has shown that NPC is closely associated with Epstein-Barr virus (EBV) infection [Miller, 1990]. Several species of antibody against EBV antigens seem to be useful for detection of NPC. Seroepidemiological studies indicated that the titre of IgA antibody against the EBV viral capsid antigen (VCA) was high in NPC patients [Henle and Henle, 1976]. In addition, this antibody was useful for the early detection of NPC in a field survey in China [Zeng et al., 1982, 1983]. However, 15–20% of NPC patients are not identified using this antibody [Sam et al., 1989]. The level of antibody neutralising EBV DNase activity was found to increase in the sera of NPC patients [Cheng et al.,

Contract grant sponsor: Department of Health, Executive Yuan, Taipei, Taiwan, Republic of China; Contract grant numbers: DOH80-TD-015, DOH81-TD-046, DOH82-TD-095, DOH84-TD-028.

*Correspondence to: Jen-Yang Chen, Graduate Institute of Microbiology, College of Medicine, National Taiwan University, No. 1, Sec. 1, Jen-Ai Rd., Taipei 10018, Taiwan, Republic of China.

Accepted 4 February 1997

1980]. Moreover, the presence of DNase neutralising antibody was demonstrated in NPC patients before the appearance of clinical symptoms [Chen et al., 1985a; 1989]. Antibody neutralising EBV DNA polymerase (DP) activity was also reported to increase in NPC patients and could be detected in most stage I patients [Liu et al., 1989]. Antibody against the EBV major DNA binding protein (MDBP) has been shown to be useful for the diagnosis of acute EBV infection [Gorgievski-Hrisoho et al., 1990]. Whether these two latter antibodies are suitable as markers for the early detection of NPC has not been determined. So far, no single serological marker has been identified as capable of screening all NPC patients. It seems plausible that testing of a panel of anti-EBV antibodies is necessary for early detection and screening of NPC. However, it remains to be determined what combination of assays is suitable to detect all NPC patients.

To assess the prognostic value of each of the above antibodies for detection of NPC, and to determine which combination is suitable for screening and early detection of the tumour, we conducted a serological study in which antibodies to EBV VCA, DNase, DP, and MDBP were evaluated. In this study, sera from NPC outpatients and from patients with tumours which became evident during the follow-up period of a prospective study were tested for EBV antibodies.

MATERIALS AND METHODS

Sera

One hundred serum specimens were obtained from histologically confirmed NPC patients who were diagnosed at the Department of Otorhinolaryngology and who had received no prior treatment. The NPC stages of these patients varied from I to V, based on the classification of Hsu et al. [1982], which is used by the National Taiwan University Hospital. Between 1983 and 1986, in a prospective study 9,869 subjects from NPC high risk areas were examined for anti-VCA using an indirect immunofluorescence assay (FA) and for anti-DNase using a neutralisation assay (NT), as described in detail in a previous report [Chen et al., 1989]. Those who were positive for anti-VCA and/or anti-DNase antibodies were examined by physicians in our ear, nose, and throat (ENT) clinic, and suspected cases were further confirmed by pathological examination. Additionally, all the subjects who were not diagnosed with NPC at recruitment were followed up by reviewing the national cancer register each year. Twenty individuals were diagnosed with NPC by May 1995, of whom 12 were reported previously [Chen et al., 1989]. Since these 20 cases were identified from the field survey by extensive screening, their sera are designated as from "screened NPC patients" in this report. All sera were inactivated at 56°C for 30 min and stored at −20°C prior to testing for anti-EBV antibodies.

Preparation of EBV Antigens

EBV antigens VCA, DNase, and DP were prepared from P3HR1 cells which were seeded at 10^6 cells per ml in RPMI 1640 medium containing 10% heat-inactivated foetal bovine serum. Then 5-iodo-2′-deoxyuridine (IdUrd) was added to P3HR1 cells at a final concentration of 60 μg/ml to induce EBV gene expression. After 3 days of incubation at 37°C, the drug was removed by replenishing with fresh medium and the cells were cultured for 1 more day. The cells were harvested and washed with phosphate buffered saline. For anti-VCA antibody detection, the cells were fixed on slides by acetone [Henle and Henle, 1966]. For determining antibodies neutralising DNase and DP activities, the IdUrd treated cells were resuspended in extraction buffer (50 mM Tris-HCl, pH 7.5, 0.3 M KCl, 5 mM β-mercaptoethanol, 0.7 mM phenylmethylsulfonyl fluoride, and 20% glycerol) and frozen and thawed 3 times. The lysate was spun and the supernatant collected as the enzyme source for DNase/NT and DP/NT.

Recombinant EBV DNase and MDBP were prepared as antigens for enzyme-linked immunosorbent assays (ELISA). Full length DNase encoded by the EBV open reading frame BGLF5 was expressed in *E. coli* and purified as described previously [Chen et al., 1993]. C-terminal MDBP (amino acids 422–1,129) was also expressed in *E. coli* and purified by ion exchange chromatography, gel filtration, and finally, SDS-PAGE, using model 491 Prep Cell (Bio-Rad Laboratories, Hercules, CA; unpublished data). Both proteins were purified to more than 90% homogeneity, as estimated by gel electrophoresis.

Determination of IgA Antibody Against EBV VCA

All sera were titrated for IgA antibody to EBV VCA by FA using the IdUrd-induced P3HR1 cells as the target. A titre of less than 1:10 was considered negative for anti-VCA antibody.

Neutralisation Test for Anti-EBV DP

Levels of antibody neutralising EBV DP activity were determined as described previously [Liu et al., 1989]. Briefly, 10μl (0.8–1.0 unit) of EBV DP was incubated with 10 μl of 10-fold diluted serum at room temperature for 20 min and then assayed for polymerase activity. The reaction mixture for the DP assay contained 50 mM Tris-HCl, pH8.0, 4 mM $MgCl_2$, 100 mM $(NH4)_2SO_4$, 0.5 mM dithiothreitol, 0.2 mg/ml bovine serum albumin, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dCTP, 5 μM [^{3}H]TTP (40 mCi/μmole; 1 Ci = 37 GBq), and 15 μg of DNase I-activated calf thymus DNA. The reaction was carried out at 37°C for 30 min. One unit of DP activity was defined as the amount of enzyme that catalyzed the incorporation of 1 pmole of TMP into activated DNA per min [Ostrander and Cheng, 1980]. Anti-EBV DP activity was expressed as the units of DP activity neutralised by 1 ml of undiluted serum. A neutralisation activity less than 200 U per ml of serum was regarded as negative.

Neutralisation Test for Anti-EBV DNase

Levels of antibody neutralising EBV DNase activity were determined as described by Chen et al. [1985b]. One unit of DNase activity was defined as the amount of enzyme which converted 1 μg of double-stranded DNA to acid-soluble material in 10 min at 37°C. The level of antibody to EBV DNase activity was expressed in terms of the units of DNase activity neutralised by 1 ml serum. Neutralisation of 2 units or more DNase activity was considered specific.

Detection of Antibodies to DNase and MDBP by ELISA

Antibodies to EBV DNase also were detected by ELISA as described previously [Chen et al., 1993], with some modifications. Briefly, 0.25 μg per well purified recombinant DNase was coated onto microtitre plates. After blocking, each well was reacted with 50 μl serum at a 1:50 dilution for 1 hour at room temperature. Alkaline phosphatase conjugated anti-human IgA was used as the secondary antibody. The optical absorbance of the reactions was read at 405 nm wave length. An OD_{405} less than 0.095 was considered negative. 0.5 μg per well of recombinant MDBP was coated on plates and 1:100 dilutions of the sera were added. Bound antibody was detected by incubating with horseradish peroxidase-conjugated, goat anti-human IgG. Results were obtained by reading the OD at 450nm. The positive/negative cut-off was 0.196 or 0.250, depending on the batch of recombinant antigen.

Statistical Methods

Analysis of variance (ANOVA) was used to determine the statistical significance of the differences in antibody titres between NPC patients at various stages. The Mantel-Haenszel chi-square test was used to test the statistical significance of the trend in seropositivity rates of various antibodies for NPC patients from early to late stages.

RESULTS

Detection of Anti-EBV Antibodies in 100 NPC Outpatients

Sera from 100 histopathologically confirmed NPC patients were examined for antibodies against EBV. The presence of anti-VCA antibody was detected by FA, anti-DNase by NT and ELISA, anti-DP by NT, and anti-MDBP by ELISA. Results for the 100 patients are shown in Table I. Positivity rates for VCA/FA, DNase/NT, DNase/ELISA, MDBP/ELISA, and DP/NT were 74%, 72%, 82%, 82%, and 71%, respectively. It is clear that no one species of antibody was present in all NPC patients. Among these patients only 1 (no. 40) was negative using all 5 tests. A discrepancy was found between ELISA and NT in detecting antibody to DNase. The two assays were 71.7% in agreement and the ELISA appeared to be more sensitive than the NT. This lack of concordance was thought to be attributable to individual differences in the immune responses to various epitopes.

Correlation Between NPC Stage and EBV Antibodies

To determine whether the serological reactivities correlated with the severity of NPC, the mean titre and positivity rates for each EBV antibody were calculated for each disease stage. As shown in Table II, the mean titres of DNase (by ELISA) and DP (by NT) increased significantly with the NPC stage ($P = 0.012$ for DNase/ELISA, $P = 0.01$ for DP/NT). Only the positivity rate for anti-VCA antibody increased with the stage of development of NPC ($P = 0.029$). According to the Pearson correlation coefficient, the correlation between any 2 tests was statistically significant with the exception of MDBP by ELISA and DNase by NT ($P = 0.06$). In order to detect NPC patients as early as possible, antibody markers present in stage I patients were compared. From the data shown in Table II, DNase by ELISA gave the highest rate (89.3%) for stage I patients. This indicates that DNase antibody, detectable by ELISA, is present in most stage I NPC patients and this assay may be useful for identifying patients with tumours at early stage.

Increasing Spectrum of Antibody Species Present in Late Stage NPC Patients

The number of species of anti-EBV antibody detectable in NPC patients increased when the disease progressed beyond stage I (Table III). Only 25% of the stage I patients were positive for all 5 tests. In contrast, more than 40% of patients at later stages were positive for all 5 antibodies. The percentages tended to increase with the disease stage, but decreased a little at stage V, where only 7 patients were tested (Mantel-Haenszel test, $P = 0.061$). In general, the immune response of end stage cancer patients is poor. If the stage V patients are excluded, it is significant that more species of EBV antibody become detectable as the disease progresses ($P = 0.034$). However, individual antibody titres did not necessarily increase in parallel.

A Combination of Two Tests Increased the Rate of Detection of NPC

When the 100 NPC patients were tested for the EBV antibodies, around 20–30% were negative for each test used. Clearly, 1 test alone cannot achieve the objective of detecting all NPC patients and it seems advisable to combine different tests for early diagnosis. The positivity rates of single and paired tests are compared in Table IV. It was found that detection rate could be increased to over 85% when 2 tests were applied, and a combination of DNase/ELISA and VCA/FA improve the sensitivity to 97%. These 2 tests provide the best combination for the detection of NPC.

Anti-EBV Antibodies in the Screened NPC Patients

Detailed information about the 20 screened NPC patients is shown in Table V. Cases 1–11 had no history of NPC at the time their blood was taken and these sera were assumed to have been collected at a "pre-NPC" stage (i.e., when an affected person feels well before the clinical onset of NPC). Cases 1–6 were un-

TABLE I. Antibodies to EBV-Specific Antigens in Sera of 100 NPC Patients*

No.	NPC stage	VCA FA	DNase NT	DNase ELISA	MDBP ELISA	DP NT
1	II	0 (–)	6.4 (+)	0.161 (+)	0.411 (+)	267 (+)
2	I	0 (–)	1.0 (–)	0.114 (+)	0.974 (+)	24 (–)
3	I	0 (–)	5.1 (+)	0.108 (+)	0.670 (+)	214 (+)
4	I	0 (–)	3.2 (+)	0.153 (+)	0.308 (+)	225 (+)
5	I	1:10 (+)	6.3 (+)	0.504 (+)	0.693 (+)	873 (+)
6	V	1:160 (+)	6.7 (+)	0.546 (+)	0.323 (+)	951 (+)
7	IV	1:10 (+)	1.4 (–)	0.115 (+)	1.068 (+)	–117 (–)
8	IV	0 (–)	0.2 (–)	0.465 (+)	0.192 (–)	561 (+)
9	IV	1:10 (+)	9.2 (+)	0.305 (+)	0.753 (+)	371 (+)
10	I	1:10 (+)	5.8 (+)	0.328 (+)	0.853 (+)	900 (+)
11	III	1:40 (+)	9.3 (+)	0.502 (+)	0.223 (–)	621 (+)
12	II	1:10 (+)	8.7 (+)	0.143 (+)	0.384 (+)	671 (+)
13	II	1:10 (+)	2.5 (+)	0.213 (+)	1.120 (+)	226 (+)
14	I	1:160 (+)	9.7 (+)	0.161 (+)	0.920 (+)	404 (+)
15	II	1:160 (+)	–0.4 (–)	0.068 (–)	0.889 (+)	105 (–)
16	I	0 (–)	1.1 (–)	0.472 (+)	0.349 (+)	69 (–)
17	I	1:160 (+)	3.1 (+)	0.064 (–)	0.563 (+)	86 (–)
18	II	0 (–)	–0.3 (–)	0.087 (–)	0.119 (–)	285 (+)
19	I	1:10 (+)	3.3 (+)	0.201 (+)	0.162 (–)	297 (+)
20	II	1:10 (+)	0.2 (–)	0.033 (–)	0.120 (–)	294 (+)
21	V	1:10 (+)	2.3 (+)	0.156 (+)	0.294 (+)	185 (–)
22	IV	1:10 (+)	0.3 (–)	0.137 (+)	0.913 (+)	35 (–)
23	II	1:160 (+)	0.8 (–)	0.184 (+)	0.367 (+)	310 (+)
24	I	1:160 (+)	3.3 (+)	0.503 (+)	0.670 (+)	190 (–)
25	IV	1:160 (+)	0.5 (–)	0.668 (+)	0.540 (+)	381 (+)
26	I	1:40 (+)	2.5 (+)	0.116 (+)	0.944 (+)	551 (+)
27	I	1:10 (+)	2.7 (+)	0.351 (+)	0.210 (–)	–271 (–)
28	I	1:10 (+)	2.9 (+)	0.565 (+)	0.651 (+)	354 (+)
29	II	1:10 (+)	3.6 (+)	0.222 (+)	1.056 (+)	678 (+)
30	III	1:640 (+)	2.1 (+)	0.007 (–)	1.001 (+)	874 (+)
31	II	1:10 (+)	–0.3 (–)	ND	0.329 (+)	ND
32	II	0 (–)	2.6 (+)	0.473 (+)	0.167 (–)	663 (+)
33	I	1:40 (+)	4.7 (+)	0.086 (–)	0.669 (+)	34 (–)
34	II	1:40 (+)	1.0 (–)	0.366 (+)	1.076 (+)	433 (+)
35	V	1:10 (+)	4.8 (+)	0.325 (+)	0.274 (+)	273 (+)
36	III	1:160 (+)	3.3 (+)	0.020 (–)	0.072 (–)	–182 (–)
37	III	1:160 (+)	3.8 (+)	0.262 (+)	1.187 (+)	413 (+)
38	V	1:40 (+)	–1.6 (–)	0.597 (+)	0.935 (+)	782 (+)
39	IV	1:10 (+)	2.3 (+)	0.490 (+)	0.420 (+)	–790 (–)
40	I	0 (–)	1.7 (–)	–0.034 (–)	0.193 (–)	187 (–)
41	IV	1:40 (+)	3.1 (+)	0.602 (+)	0.497 (+)	313 (+)
42	I	1:10 (+)	4.0 (+)	0.170 (+)	0.321 (+)	272 (+)
43	IV	1:160 (+)	2.8 (+)	0.138 (+)	1.057 (+)	716 (+)
44	III	1:160 (+)	1.1 (–)	0.088 (–)	1.051 (+)	709 (+)
45	I	1:40 (+)	0.8 (–)	0.599 (+)	0.758 (+)	813 (+)
46	II	1:10 (+)	1.9 (–)	0.064 (–)	0.167 (–)	29 (–)
47	I	1:160 (+)	4.9 (+)	0.605 (+)	0.552 (+)	228 (+)
48	I	0 (–)	0.6 (–)	0.270 (+)	0.324 (+)	66 (–)
49	IV	1:40 (+)	2.9 (+)	0.173 (+)	0.382 (+)	358 (+)
50	III	1:40 (+)	1.7 (–)	0.175 (+)	0.375 (+)	528 (+)
51	II	1:160 (+)	6.2 (+)	0.575 (+)	1.124 (+)	859 (+)
52	I	0 (–)	4.8 (+)	0.116 (+)	0.401 (+)	198 (–)
53	IV	1:640 (+)	4.0 (+)	0.664 (+)	1.232 (+)	882 (+)
54	IV	1:640 (+)	5.4 (+)	0.955 (+)	1.124 (+)	578 (+)
55	IV	1:160 (+)	6.1 (+)	0.023 (–)	0.678 (+)	652 (+)
56	IV	1:160 (+)	8.5 (+)	0.381 (+)	1.279 (+)	821 (+)
57	III	1:40 (+)	2.8 (+)	–0.043 (–)	0.008 (–)	–27 (–)
58	I	0 (–)	–1.0 (–)	0.238 (+)	0.006 (–)	187 (–)
59	III	1:640 (+)	5.3 (+)	0.468 (+)	0.367 (+)	842 (+)
60	II	1:160 (+)	3.0 (+)	0.164 (+)	1.190 (+)	542 (+)
61	I	0 (–)	0.3 (–)	0.180 (+)	0.835 (+)	197 (–)
62	I	1:10 (+)	1.2 (–)	0.366 (+)	0.538 (+)	295 (+)
63	IV	1:10 (+)	4.2 (+)	0.572 (+)	0.472 (+)	418 (+)
64	II	1:40 (+)	3.0 (+)	0.320 (+)	0.250 (+)	186 (–)
65	I	0 (–)	6.0 (+)	0.170 (+)	0.838 (+)	–79 (–)
66	V	0 (–)	1.7 (–)	0.155 (+)	0.975 (+)	147 (–)
67	II	0 (–)	4.3 (+)	0.102 (+)	0.253 (+)	347 (+)
68	II	1:10 (+)	7.1 (+)	0.110 (+)	0.929 (+)	750 (+)
69	IV	1:10 (+)	4.3 (+)	0.570 (+)	1.089 (+)	642 (+)

(continued)

TABLE I. Continued

No.	NPC stage	VCA FA	DNase NT	DNase ELISA	MDBP ELISA	DP NT
70	II	0 (–)	2.6 (+)	0.105 (+)	0.732 (+)	530 (+)
71	I	0 (–)	0.5 (–)	0.130 (+)	0.475 (+)	–181 (–)
72	III	1:40 (+)	3.0 (+)	0.296 (+)	1.038 (+)	538 (+)
73	II	1:160 (+)	5.8 (+)	0.626 (+)	1.249 (+)	746 (+)
74	IV	1:640 (+)	9.5 (+)	0.647 (+)	0.768 (+)	613 (+)
75	III	1:40 (+)	6.9 (+)	0.569 (+)	1.045 (+)	307 (+)
76	II	1:40 (+)	0.8 (–)	0.082 (–)	0.140 (–)	547 (+)
77	IV	0 (–)	5.2 (+)	0.084 (–)	0.321 (+)	393 (+)
78	IV	1:160 (+)	8.1 (+)	0.411 (+)	1.141 (+)	584 (+)
79	III	0 (–)	7.2 (+)	0.119 (+)	0.090 (–)	45 (–)
80	IV	0 (–)	–0.7 (–)	0.176 (+)	0.231 (–)	–158 (–)
81	II	1:10 (+)	5.8 (+)	0.088 (–)	0.626 (+)	374 (+)
82	III	1:40 (+)	6.5 (+)	0.399 (+)	0.813 (+)	489 (+)
83	II	1:640 (+)	9.0 (+)	0.121 (+)	0.276 (+)	209 (+)
84	III	1:40 (+)	3.6 (+)	0.602 (+)	0.591 (+)	521 (+)
85	IV	0 (–)	0.4 (–)	0.069 (–)	0.264 (+)	593 (+)
86	II	1:10 (+)	2.0 (+)	0.102 (+)	0.828 (+)	370 (+)
87	IV	1:10 (+)	6.4 (+)	0.358 (+)	0.154 (–)	581 (+)
88	V	0 (–)	3.2 (+)	0.079 (–)	0.269 (+)	–281 (–)
89	I	1:10 (+)	4.3 (+)	0.106 (+)	0.211 (–)	–205 (–)
90	III	1:160 (+)	7.7 (+)	0.343 (+)	0.999 (+)	498 (+)
91	II	1:40 (+)	6.6 (+)	0.415 (+)	0.966 (+)	385 (+)
92	I	0 (–)	8.3 (+)	0.257 (+)	0.772 (+)	364 (+)
93	II	0 (–)	7.1 (+)	0.240 (+)	0.924 (+)	311 (+)
94	I	0 (–)	4.7 (+)	0.220 (+)	0.531 (+)	173 (–)
95	III	1:10 (+)	9.5 (+)	0.266 (+)	0.569 (+)	597 (+)
96	IV	1:10 (+)	7.1 (+)	0.292 (+)	0.186 (–)	421 (+)
97	II	1:40 (+)	0.7 (–)	0.055 (–)	0.341 (+)	154 (–)
98	IV	1:40 (+)	8.3 (+)	0.466 (+)	0.709 (+)	719 (+)
99	IV	1:10 (+)	3.0 (+)	0.218 (+)	0.328 (+)	421 (+)
100	V	1:40 (+)	10.5 (+)	0.631 (+)	0.673 (+)	563 (+)
Positivity rate (%)		74	72	82	82	71

*The cut-offs are 1:10 for anti-VCA/FA, 2.0 units neutralized/ml serum for anti-DNase/NT, 0.095 for anti-DNase/ELISA, 0.250 for anti-MDBP/ELISA, and 200 units neutralized/ml serum for DP/NT antibody. (+), positive result; (–), negative result; ND, not done.

TABLE II. Mean Titres and Positivity Rates of Anti-EBV Antibodies in Sera From NPC Patients at Different Stages

		VCA FA		DNase NT		DNase ELISA		MDBP ELISA		DP NT	
Stage	No.	M[a]	R[b]	M	R	M	R	M	R	M	R
I	28	1.4	53.6	3.5	67.9	0.26	89.3	0.55	82.1	257.2	46.4
II	26	1.5	76.9	3.5	65.4	0.20	72.0	0.62	80.8	410.8	84.0
III	15	1.9	93.3	4.9	86.7	0.27	73.3	0.63	73.3	465.5	80.0
IV	24	1.7	83.3	4.3	75.0	0.37	87.5	0.66	83.3	460.5	83.3
V	7	1.5	71.4	4.2	71.4	0.36	85.7	0.53	100.0	414.4	57.1
P value*		0.321	0.029	0.180	0.427	0.012	0.971	0.516	0.541	0.010	0.056

[a]M: geometric mean of log (serum dilution fold) for VCA/FA, arithmetic means elsewhere. Units of M for the 5 tests: serum dilution fold for anti-VCA/FA, DNase units neutralized/ml serum for anti-DNase/NT, OD405nm for anti-DNase/ELISA, OD450nm for anti-MDBP/ELISA, and DP units neutralized/ml serum for DP/NT antibody.
[b]R: positivity rates (percent).
**P* values for M and R with NPC stages were based on analysis of variance (ANOVA) test and Mantel-Haenszel chi-square test for linear trend, respectively.

conscious of any clinical symptoms prior to diagnosis at NPC stages II or III. The duration between the detection of EBV antibodies and the diagnosis of NPC varied from months to years, indicating that anti-EBV antibodies were elevated in advance of the appearance of symptoms. The interval shown by case 6 was 6 years; he received radiotherapy immediately after diagnosis and the disease is still under control. Cases 7–13 were discovered by reviewing the cancer register, where they were recorded as having died of NPC. Therefore, the NPC stages are not available for this group. The exact year of diagnosis of NPC are not available for cases 12 and 13. Case 12 died 5 years after his anti-VCA was detected, and case 13 died of NPC the year after his serum was taken. Seven subjects (cases 14–20) were NPC patients with recurrent or persistent tumours or were in remission. These 20 screened NPC patients were all positive for anti-VCA and/or anti-DNase antibodies except case 7, who was negative for both antibodies.

To assess the value of the 5 tests for early detection of NPC, DNase/ELISA, DP/NT, and MDBP/ELISA

TABLE III. Number of Tests Positive for NPC Patients at Different Stages

Number of tests[a] positive	NPC stage I	II	III	IV	V	Total
0	1 (3.6%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1.0%)
1	1 (3.6%)	2 (8%)	0 (0%)	1 (4.2%)	0 (0%)	4 (4.1%)
2	5 (17.9%)	4 (16%)	3 (20%)	2 (8.3%)	2 (28.6%)	16 (16.2%)
3	7 (25%)	1 (4%)	1 (6.7%)	3 (12.5%)	0 (0%)	12 (12.1%)
4	7 (25%)	8 (32%)	3 (20%)	5 (20.8%)	2 (28.6%)	25 (25.3%)
5	7 (25%)	10 (40%)	8 (53.3%)	13 (54.2%)	3 (42.9%)	41 (41.4%)
Sample tested	28	25	15	24	7	99[b]

[a]The tests applied and their sensitivities are VCA/FA, 74%; DNase/NT, 72%; DNase/ELISA, 82%; MDBP/ELISA, 82% and DP/NT, 71%.
[b]One of the 100 NPC outpatients was excluded because the serum was not examined by all 5 tests.

TABLE IV. Antibody Positivity Rates for NPC Patients Detected by Single or Paired Tests

Tests applied		Positivity rate (%) NPC[a] screening	pre-NPC[b] detection
VCA (FA)	—	74	71
DNase (NT)	—	72	86
DNase (ELISA)	—	82	86
MDBP (ELISA)	—	82	57
DP (NT)	—	71	86
VCA (FA)	DNase (NT)	88	100
VCA (FA)	DNase (ELISA)	97	86
VCA (FA)	MDBP (ELISA)	94	71
VCA (FA)	DP (NT)	87	86
DNase (NT)	DNase (ELISA)	91	100
DNase (NT)	MDBP (ELISA)	92	100
DNase (NT)	DP (NT)	86	100
DNase (ELISA)	MDBP (ELISA)	93	86
DNase (ELISA)	DP (NT)	91	86
MDBP (ELISA)	DP (NT)	91	86

[a]Based on data from the 100 NPC outpatients.
[b]Based on data from the 7 "pre-NPC" patients detected in a prospective study (Table V).

tests were used to analyse these screened NPC patients retrospectively. Data from these individuals are shown in Table V; regrettably, sera from cases 4, 5, 7, 11, and 12 were not available. All the screened NPC patients were shown to be seropositive for EBV except case 7, emphasizing the strong association of EBV with NPC. Sera from cases 1–11 are valuable for looking at the EBV antibody status immediately prior to the development of NPC. Of these 11 "pre-NPC" cases, 7 were positive for anti-VCA and 9 for anti-DNase (NT); 6 of 7 were positive for anti-DNase (ELISA), 4 of 7 for anti-MDBP, and 6 of 7 for anti-DP. To compare the sensitivity of the 5 assays for detecting "pre-NPC" patients, data were considered only from the sera examined by all 5 tests. Thus positivity rates were calculated to be 71% (5/7) for anti-VCA, 86% (6/7) for anti-DNase (NT), 86% (6/7) for anti-DNase (ELISA), 57% (4/7) for anti-MDBP, and 86% (6/7) for anti-DP antibody (Table IV). Detection of anti-DNase antibody by NT appeared to be one of the most sensitive methods for detecting "pre-NPC" patients. Moreover, when it was combined with any other test, the detection rate could reach 100%. DNase/NT antibodies remained positive in the 5 NPC patients in remission (cases 16–20).

DISCUSSION

A number of species of EBV-associated antibody have been shown to increase in NPC patients, including anti-VCA [Henle et al., 1970], anti-EA–D [Henle et al., 1973], and antibodies against EBV-encoded enzymes, such as DNase [Cheng et al., 1980], DP [Tan et al., 1986], and thymidine kinase [Gabhann et al., 1984]. Anti-VCA/IgA, anti-EA–D/IgA, and anti-DNase have been demonstrated in mass serological surveys and follow-up studies to be valuable markers for early detection of NPC [Zeng et al., 1982, 1983; Deng et al., 1995; Chen et al., 1989]. In this study, we compared the efficiency of antibodies against VCA, DNase, MDBP, and DP for detection of NPC. In addition to NPC outpatients, we also investigated the "screened NPC patients," including 7 treasured "pre-NPC" sera collected from a cohort study carried out in an NPC high-risk area in Taiwan. Although this number is small, it represents sera collected before the onset of NPC with a follow-up of more than 10 years. The maximal detection rates are summarised in Table VI. DNase/ELISA gave a significant result not only for NPC at a late stage but also for patients at an early stage and even at the earlier "pre-NPC" stage. This suggests that there was constant expression of EBV DNase in individuals once they were affected by NPC. When DNase/ELISA was combined with other tests, the detection rates improved significantly. Therefore we recommend the use of DNase/ELISA in combination with other tests for NPC detection.

For patients in remission, we looked for antibody with a low positivity rate and low titre in order to reflect the patient's status. A previous observation was that DP antibody declined in treated patients [Liu et al., 1989]. From the current work and other unpublished data, we find that DP/NT showed a lower positivity rate in patients in remission. In particular, we had consecutive sera from one patient (case 6, Table V) which covered the whole course of NPC development, including "pre-NPC," early NPC, and remissive stages. He was diagnosed with NPC in 1992, and his sera from 1986, 1990, 1991, and 1992 were fortunately available. We checked his sera for anti-DP antibody and found that the antibody level appeared to increase gradually, reaching a maximum before the diagnosis of NPC, and

TABLE V. Detection of Anti-EBV Antibodies by Five Serological Tests in NPC Patients Found in a Prospective Study*

NPC patients	Time of blood collection	VCA FA	DNase NT	DNase ELISA	MDBP ELISA	DP NT	Clinical status (at the time of visit to ENT clinic)	Year diagnosed with NPC
1	1984.9	1:160 (+)	6.2 (+)	0.107 (+)	0.655 (+)	1020 (+)	Stage II	1985
2	1984.10	1:160 (+)	8.7 (+)	0.357 (+)	0.332 (+)	900 (+)	Stage III	1985
3	1987.5	1:160 (+)	4.5 (+)	0.140 (+)	0.078 (–)	440 (+)	Stage III	1987
4	1985.9	1:40 (+)	8.5 (+)	ND	ND	ND	Stage II	1986
5	1985.9	1:40 (+)	5.9 (+)	ND	ND	ND	Stage II	1985
6	1986.12	0 (–)	4.7 (+)	0.125 (+)	0.032 (–)	258 (+)	Stage II	1992
7	1984.9	0 (–)	(–)	ND	ND	ND	NA	1986
8	1984.9	1:160 (+)	(–)	0.688 (+)	0.542 (+)	340 (+)	NA	1990
9	1984.9	0 (–)	5.1 (+)	0.054 (–)	0.053 (–)	(–)	NA	1990
10	1985.1	1:160 (+)	6.6 (+)	0.373 (+)	0.976 (+)	540 (+)	NA	1986
11	1985.9	0 (–)	8.1 (+)	ND	ND	ND	NA	1992
12	1984.12	1:40 (+)	1.5 (–)	ND	ND	ND	NA	1989[a]
13	1984.10	1:10 (+)	7.9 (+)	0.088 (–)	1.229 (+)	87 (–)	NA	1985[a]
14	1985.9	0 (–)	5.5 (+)	0.023 (–)	0.241 (+)	122 (–)	Recurrent 1986.6	1984
15	1984.10	1:160 (+)	8.9 (+)	0.120 (+)	0.470 (+)	380 (+)	Persistent 1985.7	1979
16	1984.10	1:10 (+)	3.2 (+)	0.000 (–)	0.011 (–)	1060 (+)	Remission 1985.2	1977
17	1984.11	0 (–)	6.6 (+)	0.109 (+)	0.183 (–)	(–)	Remission 1985.5	1967
18	1984.11	1:10 (+)	5.9 (+)	0.550 (+)	0.207 (+)	350 (+)	Remission 1985.4	1982
19	1984.10	0 (–)	3.4 (+)	0.015 (–)	0.174 (–)	(–)	Remission 1985.2	1981
20	1985.1	1:40 (+)	2.6 (+)	0.521 (+)	0.706 (+)	(–)	Remission 1985.7	1983

*Cut-offs are 1:10 for anti-VCA/FA, 2.0 units neutralized/ml serum for anti-DNase/NT, 0.095 for anti-DNase/ELISA, 0.196 for anti-MDBP/ELISA, and 200 units neutralized/ml serum for DP/NT antibody.
[a]Year died of NPC.
NA, not available. ND, not done.

TABLE VI. The Most Efficient Tests for Detection of NPC Patients at Various Stages

Stages	Sample tested	Maximal detection rate			
		One test		Paired tests	
Pre-NPC	7	86%	DNase/ELISA DNase/NT DP/NT	100%	DNase/NT + DNase/ELISA DNase/NT + VCA/FA DNase/NT + DP/NT DNase/NT + MDBP/ELISA
Early NPC (stage I)	28	89%	DNase/ELISA	96%	DNase/ELISA + VCA/FA DNase/ELISA + DNase/NT DNase/ELISA + MDBP/ELISA
NPC (stages I–V)	100	82%	DNase/ELISA MDBP/ELISA	97%	DNase/ELISA + VCA/FA

TABLE VII. Anti-EBV Antibodies in a Long-Term Follow-Up NPC Patient

Date	DP NT	DNase NT	VCA FA	Clinical status
1986.12	258(+)	4.7(+)	(–)[a]	normal
1990	424(+)	7.6(+)	1:640(+)	normal
1991	1000(+)	6.0(+)	1:160(+)	normal
1992.6	NA	NA	NA	diagnosed as NPC stage II
1992.6–7	NA	NA	NA	radiotherapy treatment
1992.12	(–)	5.8(+)	1:160(+)	remission

[a]negative result NA, not available.

returning to negative after therapy (Table VII). The results support the view that neutralising antibody to DP activity may have potential as a marker for early detection and prognosis of NPC.

MDBP antibody was demonstrated to be useful for diagnosing acute EBV infection [Gorgievski-Hrisoho et al., 1990]. In Taiwan, most people are infected by EBV in early childhood [Tsai et al., 1989; Liu et al., 1992] and primary infections of adults are very rare. We used ELISA to detect MDBP antibody in NPC patients to determine its value for detection of the tumour in adults and most NPC patients (82%) were found positive. In a recent study, a mass serological survey of the anti-MDBP antibody in healthy donors was undertaken and the positivity rate was about 12% (manuscript in preparation). When this antibody was examined in the "pre-NPC" sera, the positivity rate was not high (57%). However, the antigen we used was a truncated version of MDBP, and this may account for the low detection rate for "pre-NPC" patients. The data obtained here suggest that MDBP antibody is more suitable to aid clinical diagnosis of suspected NPC patients.

Tests for the early detection of tumours are essential if survival rates are to be improved. NPC patients usually develop high levels of antibodies against EBV and these antibodies have proved useful in early diagnosis. However, to be useful for mass screening in high-risk areas, such as Taiwan, a very sensitive and specific test

is required to avoid screening out too many individuals from further follow-up. The sensitivities of the 5 tests employed in this study were calculated to be 74% for VCA/FA, 72% for DNase/NT, 82% for DNase/ELISA, 82% for MDBP/ELISA, and 71% for DP/NT. In previous studies, the specificities of these 5 tests were reported to be more than 95% for VCA/FA [Henle and Henle, 1976], 94.7% for DNase/NT [Chen et al., 1987], 100% for DNase/ELISA [Chen et al., 1993], 82% for MDBP (unpublished data), and 93.5% for DP/NT [Liu et al., 1989], respectively. In our recent study, 13,411 sera from NPC high risk area were also examined by the 5 tests. The specificities proved to be 97.6% for VCA/FA, 88.1% for DNase/NT, 87.9% for DNase/ELISA, 87.7% for MDBP/ELISA, and 94% for DP/NT (manuscript in preparation). Calculation of sensitivity and specificity of a test depends on sample size and variations among different subjects tested. Discrepancies may be found between surveys including large versus limited numbers of subjects. Care must be taken before drawing any conclusions regarding the specificity and sensitivity of a test, although we suggest that DNase/ELISA was the most sensitive test for detecting "pre-NPC" cases. Finally, the results obtained from this evaluation of multiple antibodies to EBV as markers for detecting NPC suggests that a combination of at least 2 assays would give better results in screening for early cases.

ACKNOWLEDGMENTS

The authors thank Dr. T.J. Harrison for reading and providing helpful comments on this manuscript. This study was supported by grants DOH80-TD-015, DOH81-TD-046, DOH82-TD-095, and DOH84-TD-028 from Department of Health, Executive Yuan, Taipei, Taiwan, Republic of China.

REFERENCES

Chen JY, Hwang LY, Beasley RP, Chien CS, Yang CS (1985a): Antibody response to Epstein-Barr virus–specific DNase in 13 patients with nasopharyngeal carcinoma in Taiwan: A retrospective study. Journal of Medical Virology 16:99–105.

Chen JY, Liu MY, Chen CJ, Hsu MM, Tu SM, Lee HH, Kou SL, Yang CS (1985b): Antibody to Epstein-Barr virus–specific DNase as a marker for the early detection of nasopharyngeal carcinoma. Journal of Medical Virology 17:47–49.

Chen JY, Chen CJ, Liu MY, Cho SM, Hsu MM, Lynn TC, Shieh T, Tu SM, Lee HH, Kuo SL, Lai MY, Hsieh CY, Hu CP, Yang CS (1987): Antibodies to Epstein-Barr virus–specific DNase in patients with nasopharyngeal carcinoma and control groups. Journal of Medical Virology 23:11–21.

Chen JY, Chen CJ, Liu MY, Cho SM, Hsu MM, Lynn TC, Shieh T, Tu SM, Beasley RP, Hwang LY, Lee HH, Kuo SL, Yang CS (1989): Antibody to Epstein-Barr virus–specific DNase as a marker for field survey of patients with nasopharyngeal carcinoma in Taiwan. Journal of Medical Virology 27:269–273.

Chen JY, Liu MY, Hsu TY, Cho SM, Yang CS (1993): Use of bacterially expressed antigen for detection of antibodies to the EBV-specific deoxyribonuclease in sera from patients with nasopharyngeal carcinoma. Journal of Virological Methods 45:49–66.

Cheng YC, Chen JY, Glaser R, Henle W (1980): Frequency and levels of antibodies to Epstein-Barr virus–specific DNase are elevated in patients with nasopharyngeal carcinoma. Proceedings of the National Academy of Sciences USA 77:6162–6165.

Deng H, Zeng Y, Lei Y, Zhao Z, Wang P, Li B, Pi Z, Tan B, Zheng Y, Pan W (1995): Serological survey of nasopharyngeal carcinoma in 21 cities of South China. Chinese Medical Journal 108:300–303.

Gabhann PM, Sugawara K, Ito Y (1984): Characterization of Epstein-Barr virus–related thymidine kinase induced in nonproducer cells by superinfection or chemical treatment. Intervirology 21:104–109.

Gorgievski-Hrisoho M, Hinderer W, Nebel-Schickel H, Horn J, Vornhagen R, Sonneborn HH, Wolf H, Siegl G (1990): Serodiagnosis of infectious mononucleosis by using recombinant Epstein-Barr virus antigens and enzyme-linked immunosorbent assay technology. Journal of Clinical Microbiology 28:2305–2311.

Henle G, Henle W (1966): Immunofluorescence in cells derived from Burkitt's lymphoma. Journal of Bacteriology 91:1248–1256.

Henle G, Henle W (1976): Epstein-Barr virus specific IgA serum antibodies as an outstanding feature of nasopharyngeal carcinoma. International Journal of Cancer 17:1–7.

Henle W, Henle G, Ho HC, Burtin P, Cachin Y, Clifford P, DeSchryver A, De The G, Diehl V, Klein G (1970): Antibodies to Epstein-Barr virus in nasopharyngeal carcinoma, other head and neck neoplasms, and control groups. Journal of National Cancer Institute 44:225–231.

Henle W, Ho HC, Henle G, Kwan HC (1973): Antibodies to Epstein-Barr virus–related antigens in nasopharyngeal carcinoma. Comparison of active cases with long-term survivors. Journal of National Cancer Institute 51:361–369.

Hsu MM, Huang SC, Lynn TC, Hsieh T, Tu SM (1982): The survival of patients with nasopharyngeal carcinoma. Otolaryngology—Head & Neck Surgery 90:289–295.

Liu MY, Chou WH, Nutter L, Hsu MM, Chen JY, Yang CS (1989): Antibody against Epstein-Barr virus DNA polymerase activity in sera of patients with nasopharyngeal carcinoma. Journal of Medical Virology 28:101–105.

Liu MY, Cho SM, Tsai WS, Chang MH, Chen JY, Yang CS (1992): Prevalence of antibody to Epstein-Barr virus DNase in children. Intervirology 34:192–196.

Miller G (1990): Epstein-Barr virus, biology, pathogenesis, and medical aspects. In Fields BN, Knipe DM, Chanock RM, Hirsch MS, Melnick JL, Monath TP, Roizman B (eds): "Virology." New York: Raven Press, Ltd., pp 1921–1957.

Ostrander M, Cheng YC (1980): Properties of herpes simplex virus type 1 and type 2 DNA polymerase. Biochemica et Biophysica Acta 609:232–245.

Sam CK, Prasad U, Pathmanathan R (1989): Serological markers in the diagnosis of histopathological types of nasopharyngeal carcinoma. European Journal of Surgical Oncology 11:17–20.

Shanmugaratnam K (1982): Nasopharynx. In Schottenfeld D, Fraumeni JF Jr (eds): "Cancer Epidemiology and Prevention." Philadelphia: W.B. Saunders Company, pp 536–553.

Tan RS, Li JS, Grill SP, Nutter LM, Cheng YC (1986): Demonstration of Epstein-Barr virus specific DNA polymerase in chemically induced Raji cells and its antibody in serum from patients with nasopharyngeal carcinoma. Cancer Research 46:5024–5028.

Tsai WS, Chang MH, Chen JY, Lee CY, Liu YG (1989): Seroepidemiological study of Epstein-Barr virus infection in children in Taipei. Acta Pediatrica Sinica 30:81–86.

Yeh S (1985): Survey of cancer in Taiwan. In Chang EH, Lin JK, Huang PC (eds): "Molecular Biology of Neoplasia.", Taipei: Academia Sinica, pp 23–31.

Zeng Y, Zhang LG, Li HY, Jan MG, Zhang Q, Wu YC, Wang YS, Su GR (1982): Serological mass survey for early detection of nasopharyngeal carcinoma in Wuzhou city, China. International Journal of Cancer 29:139–141.

Zeng Y, Zhong JM, Li LY, Wang PZ, Tang H, Ma YR, Zhu JS, Pan WJ, Liu YX, Wei ZN, Chen JY, Mo YK, Li EJ, Tan BF (1983): Follow-up studies on Epstein-Barr virus IgA/VCA antibody-positive persons in Zangwu County, China. Intervirology 20:190–194.